United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Linking thyroid hormone signaling to brain development using gene editing in zebrafish
LAPR Number:	19-04-002
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 RTP/USEPA/US
Date Originated:	11/24/2015
LAPR Expiration Date:	04/30/2019
Agenda Date:	01/13/2016
Date Approved:	04/05/2016
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 /RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	04/05/2016	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	04/05/2016	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Linking thyroid hormone signaling to brain development using gene editing in zebrafish

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
CSS 17.02Virtual Tissue Models Task 4 – Thyroid Hormone Dynamics in the Neurovascular Unit (NVU), Output 4c – Zebrafish developmental model of the NVU.

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/ISTD/SB/[redacted]/2015-02-r0

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* Exemption 6 Exemption 6 [redacted] RTP/USEPA/US	ISTD *Branch* SBB	MD

4. Alternate Contact:

Alternate Contact	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US	ISTD *Branch* GCTB	MD

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

Thyroid hormones play a critical role in vertebrate development. Genes that regulate the synthesis and metabolism of thyroid hormones are well conserved across vertebrates, including zebrafish. However, the effects of abnormal thyroid hormone signaling on brain development are not well defined.

Reduced thyroid hormone during pregnancy results in cognitive effects and hearing loss in humans. Many environmental chemicals are thought to elicit toxicity via disruption of thyroid hormone signaling. The mechanism by which environmental chemicals cause nervous system toxicity is not known. This study will precisely map out the effect of each critical thyroid hormone gene and its effects on BBB and lateral line and neurobehavioral development in zebrafish. This data is critical to ascertain the identity of thyroid hormone genes that are most sensitive to disruption by environmental chemicals. This will enable the prioritization of thyroid hormone specific mechanisms by which environmental chemicals may cause toxicity during development.

In summary, this research aims to characterize the effects of genetic TH system dysfunction on BBB and lateral line development and T3/T4 generation in zebrafish. The effects of genetic mutations will also be compared to the effects of classic thyroid disrupting chemicals

Overall, this protocol outlines an in vivo approach to generate thyroid hormone system zebrafish mutants that lack genes encoding proteins that control thyroid hormone synthesis, transport, and metabolism. This protocol outlines a genetic strategy to generate thyroid hormone mutants and a number of assays that will be developed and considered in order to link thyroid hormone signaling to adverse organismal outcomes – specifically related to nervous system development and function. Endpoint-wise, we will examine thyroid hormone-dependent neurovascular development including blood brain barrier (BBB) development and formation of the lateral line in zebrafish. The lateral line is a system of sensory cells found in fish that detect movement and vibration in the surrounding water that is analogous to the inner ear in fish. Lateral line development is dependent on thyroid hormone signaling. We will also quantify thyroid hormone levels in zebrafish mutants. Overall, this protocol will enable the discovery of thyroid hormone genes that, when disrupted, change the course of normal nervous system development and function.

The data generated from these studies will support model development outlined in 17.02 Task 3 and provide actionable data for computational models under development in 17.02 Task 4.

This research is expected to generate information on the genetics of developmental systems which depend on the presence of thyroid hormone, and lead to the normal development of the neurological system. Some environmental chemicals cause disruption in thyroid hormone dependent signaling pathways. Disruption of genes that control levels of thyroid hormone would therefore be expected to cause abnormal nervous system development and abnormal nervous system function in humans and other animals including fish. The benefits of understanding the specific effects of thyroid hormone disruption, by which many environmental chemicals are thought to exert their toxic effects, will provide novel insight into potential human and aquatic health effects of thyroid hormone disruption, and outweigh the justified concern about the use of animals in environmental health effects research.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

Animals are necessary for this research because we require a model organism that contains a nervous system, thyroid gland, and blood brain barrier. We also need a model system that allows for the use of simple behavioral assays to assess development of the neurobehavioral system. There are no in vitro systems which meet these criteria.

b. Justify the species requested:

Zebrafish is an outstanding model for genetic manipulation studies in part due to its small size, rapid development, optical transparency, embryonic development external to the mother, amenability to genetic screening, and fully defined genome. Zebrafish possess orthologs (genes in different species that evolved from a common ancestral gene) for 70% of human genes and 86% of 1318 human drug targets and thus serves as an appropriate translational model for human health and environmental toxicology. As a side note, human drug discovery and drug toxicity testing are often performed in zebrafish because human drugs will target zebrafish genes or gene products. In addition to genetic conservation, methods to generate targeted genetic mutations in zebrafish have been published. .

3. How was it determined that this study is not unnecessary duplication?

In order to assure that the research is not an unnecessary duplication, we have extensively searched PubMed to catalog all instances of gene knockdown (method to induce partial loss of gene function) by morpholino injection or complete knockout (genetic mutations that cause full loss of gene function) of thyroid hormone related genes. There are a handful of reports where 1-2 genes have been knocked out or down. This study will be the first systematic knockout of all key players in thyroid hormone synthesis and the first to delineate the effect of gene knockout on BBB and lateral line development and T3/T4 generation. The data captured in the literature search is attached to the LAPR **Exemption 6Exemption 6**

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

Overall, this protocol outlines an in vivo approach to use the CRISPR-Cas gene editing system to generate thyroid hormone system mutants. The CRISPR-Cas system is inspired by a bacterial defense system for invading viruses. Guide RNAs termed gRNAs bind to viral DNA based on sequence complementarity (A:T and C:G base binding). They also bind to the Cas 9 protein, bringing this nuclease into contact with invading virus. The Cas 9 nuclease cleaves the viral DNA, causing double stranded breaks. Error-prone processes are used to repair the breaks, often resulting in base insertions or deletion that inactivate the virus. The same concept has been applied to zebrafish to introduce inactivating mutations and thereby completely knocking out gene function at specific genetic locations.

This protocol outlines a genetic strategy to generate thyroid hormone mutants and a number of assays that will be developed and considered in order to link thyroid hormone signaling to adverse organismal outcomes. Endpoint-wise, we will examine thyroid hormone-dependent neurovascular development including blood brain barrier (BBB) development and formation of the lateral line in zebrafish. We will also quantify thyroid hormone levels in zebrafish mutants. This protocol will enable the discovery of thyroid hormone genes that, when gene function is lost, change the course of normal brain development and function.

Thyroid hormone Mutant generation.
Thyroid hormone mutants will be generated according to Varshney et al. and Shah et al (attached). Newly fertilized zebrafish embryos at the single cell stage will undergo microinjection. 1. Microinjection is a method to deliver very small volumes of liquid to each developing embryo. A small needle is injected into the yolk or the first cell of the developing fish. A small puff of air delivers the liquid. This method is used to introduce foreign genetic material that is specifically designed, in this case, to bind to and cause mutations in zebrafish genes that code for proteins that control the thyroid hormone system.

For this LAPR, zebrafish embryos will be microinjected with commercially available guide RNAs (gRNAs) and Cas9 mRNA or protein (Genecopoeia). The gRNAs will directly bind to target genes that encode proteins that control thyroid hormone levels. The gRNAs also contain a region that binds the Cas9. Cas9 is a nuclease that will cause a double stranded break in the target gene. Double stranded breaks are repaired by error-prone methods, often resulting insertions or deletions that cause premature stop codons thus generating targeted mutants.

gRNA sequences will be designed using freely available online tools including:
https://www.deskgen.com/landing/; http://www.crisprscan.org/;
http://www.e-crisp.org/E-CRISP/designcrispr.html; or http://crispr.mit.edu/. gRNAs will undergo sequence quality assurance by the vender. A scrambled gRNA (does not target zebrafish genome) will be injected into control embryos.

Mutation confirmation in embryos and larvae less than 14 days old.
The presence of desired mutations will be determined by fluorescence polymerase chain reaction (PCR) and/or sequencing in whole tissue homogenates.

Blood brain barrier (BBB) assessments in transgenic kdr1 zebrafish.
After mutants are generated in tg(kdrl:EGFP)s843/+ (AB) strain embryos, we will evaluate BBB formation. On day 0, embryos will be bleached at 3-4 hours post fertilization according to the Zebrafish Book (Westerfield 2007). Based on previous methods (Exemptio Exemptio Exemptio et al. 2014), embryos will be housed in 100 mm Petri dishes at 26 degrees C overnight in 10% Hanks' buffer. At 24 hours post fertilization, embryos will be manually dechorionated and periodically assessed for BBB formation using confocal microscopy. We will image embryos in the lateral and dorsal positions to look for structural differences between mutants and wildtype or scramble gRNA-injected controls. Prior to imaging, embryos will be anesthetized using 600 uM eugenol then imaged using a Nikon A1 laser scanning confocal microscope. Categorical differences in BBB development (normal/abnormal) will be examined.

Lateral line assessments in embryonic zebrafish.
After mutants are generated (see above), we will analyze the structure of the lateral line in wildtype or kdr1 larvae immersed in YO-PRO-1 dye according to Ou et al (attached below). YO-PRO1 selectively labels hair cell nuclei of the lateral line. On day 0, embryos will be bleached at 3-4 hours post fertilization according to the Zebrafish Book (Westerfield 2007). Based on previous methods **Exemption 6** embryos will be housed in 100 mm Petri dishes at 26 degrees C overnight in 10% Hanks' buffer. At 24-48 hours post fertilization, embryos will be manually dechorionated to assess lateral line formation using confocal microscopy. Larvae will be anesthetized using 600 uM eugenol then immersed in 1-10 uM YO-PRO1 (Invitrogen; Y3603) in 10% Hanks' for 20-60 minutes. Larvae will then be rinsed three times in 10% Hanks' prior to imaging. These methods will be performed in anesthetized wildtype Z, mutant, and transgenic kdr1 zebrafish. Categorical differences in lateral line development endpoints (normal/abnormal) or differences in number or spacing in the lateral line will be examined as potential endpoints. All data will be compared to scramble gRNA-injected controls.

T3/T4 quantification in embryonic and larval zebrafish.
To connect TH system dysfunction in mutants to neurobehavioral outcomes, we need to quantify changes in T3 and T4 levels in the developing embryo. TH extractions and hormone quantification will be performed in MED/NHEERL.

Chemical exposures.
In addition to assessing the lateral line and BBB, wildtype and kdr1 embryos and/or TH system mutants will be exposed to test compounds (methimazole, perchlorate, propylthiouracil (PTU), and thyroxine) to compare the effects of classic thyroid disrupting compounds to genetic disruption of the TH signaling system. Chlorpyrifos may occasionally be used as a positive control for behavioral experiments. Dosing will begin on Day 0 or 1 and continue through Day 5. Please note that behavior experiments will end on Day 6 but because the BBB and lateral line develop by 3-4 dpf and it is optimal to image before pigmentation becomes a barrier to imaging, some dosing experiments may end by day 3 or 4. On the final day of the experiment, all larvae will be placed in control solution.

Behavioral experiments.
At the end of all experiments on Day 6, mutant and chemically exposed larvae will be tested using the light/dark locomoter assay. For behavior testing, plates will be stored in the behavior room set to 26 degrees C for a minimum of 60 minutes before locomotor testing. We may occasionally test behavior in 14 day old zebrafish. If this occurs, all larvae will be fed rotifers and/or artemia. All larvae will be euthanized by 14 days post fertilization.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.
See attachment "20151209_Thyroid LAPR # of animals.pdf"

Overall numbers per study:
Study A: TH mutant line generation, lateral line and BBB observations: 10800 embryos
Study B: T3/T4 pilot study to determine the number of larvae necessary to quantify T3/T4: 1575
Study C: T3/T4 quantification in mutant zebrafish lines: 1800

Study D: Analysis of classic thyroid hormone disrupting chemicals: 10800
Total for Studies A-D: 24975

Justification:
Study A: For initial microinjections we will use 125 embryos per replicate per gRNA group for a total of 1500 per 12 gRNA groups (see "20151209_Thyroid LAPR # of animals.pdf "). We will also include 125 embryos for each scrambled control gRNA injection for an additional 1500 embryos per 12 groups. Therefore, the number of embryos needed for each biological replicate of 12 target gRNA and 12 scrambled control gRNA microinjection groups is 3000 embryos. We will perform three separate microinjection experiments, so 3000 X 3 = 9000 embryos. We anticipate 20% of gRNAs will need to be redesigned and therefore request an additional 1800 embryos. Therefore, the total number of embryos needed for Study A is 10800.

To provide justification for 125 embryos per gRNA per replicate in Study A, we expect that ~15% of embryos will die as an artifact of the injection process. These embryos will be removed from the study, leaving approximately 105 embryos per gRNA per biological replicate. 15 will be visually inspected for BBB development. 15 will be observed for lateral line development. 40 will be used for PCR/sequencing to detect mutations. 35 will be used for behavior assessments.

Study B: To perform a pilot study to determine how many larvae are needed for thyroid hormone quantification, we will test 3 pools of 25, 50, and 100 wildtype Z larvae at 6 dpf for a total of 175 embryos. 175 X 3 = 525 larvae for three biological replicates. We expect to optimize the extraction protocol, and anticipate completing this pilot design under three different extraction conditions for a total of 1575 larvae.

Study C: In Study C, we will quantify thyroid hormone in mutant zebrafish lines. Initially, we expect to quantify thyroid hormone levels in three groups of gRNAs injected embryos. Assuming we use 200 larvae per replicate per gRNA, we anticipate using 600 per gRNA for three biological replicates. 600 X 3 groups = 1800 embryos.

Study D: To compare mutant phenotypes observed in Study A to the effects of classic thyroid hormone disrupting chemicals, we will expose wildtype, thyroid hormone mutant, and/or kdr1 transgenic lines to thyroid disruptors. Thyroid disrupting chemicals to be used are perchlorate, PTU, methimizole, or thyroxine. We will expose 100 embryos per replicate to 8 concentrations (9, including vehicle control) = 900 embryos per replicate per chemical. For 4 chemicals and 3 biological replicates, this study requires 10800 embryos.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	*Adults*	*Offspring*
C) Minimal, transient, or no pain/distress:	0	24975
D) Potential pain/distress relieved by appropriate measures:	0	0
E) Unrelieved pain/distress:	0	0

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):
Embryos will be microinjected with Cas9 mRNA, target gRNA or scrambled control gRNA at the single cell stage on Day 0. Some embryos will be treated with < 120 uM of the test chemicals by immersion for 1-6 days post fertilization (dpf). The dosing vehicle is DMSO (dimethyl sulfoxide). Exposure solutions in DMSO will result in final vehicle concentrations of 0.4% DMSO.

b. Survival Blood Collections (method, volume, frequency):
n/a

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging

electric shock):
n/a
d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:
n/a
e. Breeding for experimental purposes (e.g. length of pairing, number of generations):
n/a
f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:
Mutant embryos and larvae will be housed in 100 mm petri dishes through 6 days post fertilization. Each petri dish will be labeled with the date of birth (DOB), target gene, and gRNA identification number (ID). If animals are transferred into 96 well plates for behavioral testing, a plate log will be maintained that indicates which petri dish each individual embryo came from. Animals will be monitored on Days 0 and 6 by an approved researcher on the LAPR. For chemical exposures in 96 well filter plates, embryos/larvae will be monitored on Days 0 and 6 by an approved researcher on the LAPR.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).
a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):
n/a
b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):
n/a
c. Testing methods:
n/a
d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):
n/a
e. Describe how animals will be monitored (e.g., frequency of observations, by whom):
n/a
f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:
n/a
g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:
n/a

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)
a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:
n/a
b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:
n/a
c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):
n/a
d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):
n/a
e. Will any animal be subject to more than one surgical procedure over the course of its lifetime,

either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

n/a

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

We do not expect to see lethargy, ataxia or tremors. We do expect to observe overt malformations.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

The hypothesis of this study is that genetic deletion of thyroid hormone gene function can alter normal brain development and function. As such, one of the endpoints we will assess is malformations. Therefore, if malformations do occur, we do not plan on stopping the experiment. The exception is that, if embryos die as a result of the microinjection process, we will remove them from the study.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

n/a

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study:		0
b. Animals to be transferred from another LAPR:		24975
LAPR Number that is the source of this transfer:	17-07-002	
c. Animals to be transferred from another source:		0
d. Offspring produced onsite (used for data collection and/or weaned):		0
e. TOTAL NUMBER of animals for duration of the LAPR		24975

2. Species (limited to one per LAPR): Danio rerio (zebrafish)

3. Strain: wildtype (Z) and transgenic kdr1:EGFP lines

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

n/a

4. Sources of animals:

Breeding protocol LAPR 17-07-002

5. Provide room numbers where various procedures will be performed on animals:

Exemption 6 Exemption 6 Exemption 6 (fish room), Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

Fish will be housed in Exemption 6 Exemption 6 Exemption 6 o Exemption 6 Exemption 6 *Room Numbers:* Exemption 6 Exemptio Exemptio Exemptio lab).

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

Embryos and larvae will be placed in sealed Petri dishes or sealed 96 well plates. Primary containers will be placed in secondary tupperware containers. These secondary containers will be transported between buildings and facilities in closed coolers.

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

In general, all zebrafish will be kept in Exemption 6 until 6 days post-fertilization, at which point they will be euthanized according to approved IACUC procedures. No fish will be returned to the A building animal facility.

All experiments will take place between 0-6 days. It is occasionally necessary to feed larvae if they are kept in the laboratory longer than 6 days; this happens very rarely, only if there is a scheduling problem or equipment malfunction.

From 7-14 days, we feed larvae either Gemma micro zebrafish feed or live rotifers. The Gemma micro is a manufactured food appropriate for young fish and is used in many zebrafish facilities. Rotifers are reared in Exemption 6 Exemption 6. The larvae are fed either Gemma micro or rotifers once in the late afternoon and once in the morning. The feedings are noted on the log sheet on the outside of the incubator and also on the log sheet that we keep with each plate detailing the plate changes and feedings if the larvae are kept in the laboratory longer than 6 days. Larvae older than 14 days will be not be kept in the laboratory; they will be euthanized.

All zebrafish will be housed in an incubator set to 26°C. Daily temperature recordings will be noted on the log sheet on the outside of the incubator. Zebrafish embryos and larva will be housed in 100 mm petri dishes, 96 well microtiter plates (1 embryo/well); 24 well microtiter plates (15 embryos/well); or 12 well plates (30 embryos/well). Water changes will occur a minimum of once every 60 hours. We arrived at 60 hours to allow for water changes on Friday evenings and Monday mornings. Data from Exemption 6 shows no difference in behavior between larvae that did or did not experience a water change over the weekend. There was also no difference in overall mortality between groups.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

We will use zebrafish embryos/larvae without enrichment. To our knowledge, there is no precedent for enrichment in embryonic/larval zebrafish toxicity assays as enrichment (typically in the form of mesh or plastic plants added to large housing chambers) would confound developmental toxicity testing petri dishes and microtiter plates.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

n/a

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

The maximum dose of any test chemical will be 120 uM by immersion of the embryos.

We will use chemicals covered by HSRP578 (see Exemption 6 list of chemicals" and "HSRP 578 Chem Screen") including: methimazole, perchlorate, propylthiouracil (PTU), and thyroxine (3, 5, 3'-triiodothyronine). At 80 uM, methimizole does not cause malformations or death (internal Exemption 6 Exemption 6 ToxCast data). Concentrations greater than 250 uM cause failure to hatch (Jomaa et al., attached). Thyroxine has a lowest observable effect level (LOEL) for malformations of 50 nM (Jomma et al., attached and Truong et al, supplemental data attached as "OSU ToxCast sup…pdf"). PTU causes failure to hatch at 400 uM (Jomaa et al.). Sodium perchlorate causes impaired swim bladder inflation at 25 uM (Jomaa et al.).

Chlorpyrifos will be used as a positive control for behavioral experiments. This compound has an half maximal concentration (AC50) of 4.24 uM for developmental toxicity in zebrafish Exemption 6 Exemption 6 internal ToxCast data).

Dimethyl sulfoxide (DMSO) will be used as a vehicle. The final concentration of DMSO will not exceed 0.4%.

The embryos may also be exposed to Tricaine as an anesthesia/euthanasia (250 mg/L with 1 gram of sodium bicarbonate added) or clove oil (eugenol 600 uM) as an anesthesia.

10% Hanks is a 1:10 dilution of Hanks' in deionized water. Full strength Hanks' is composed of 0.137 M NaCl; 5.4 mM KCl; 0.25 mM Na2H PO4; 0.44 mM KH2 PO4; 1.3 mM CaCl2; 1.0 mM Mg SO4; and 4.2 mM NaH CO3.

0.6% bleach will be used to bleach embryos prior to use.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.
For each chemical, we will use pharmaceutical grade compound, unless in the rare event that it is unavailable.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.
n/a

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.
Personnel performing the weighing of the chemical and dissolution into DMSO will wear personal protective equipment including lab coat, nitrile gloves, safety glasses, and a N95 respirator. The working solutions will be distributed into 1 ml aliquots and frozen at -80 degrees C in the Exemption 6 Exemption 6 Exemption 6 common equipment room. The working solutions may also be stored in the refrigerator in room Exemption 6. A lab coat, gloves, safety glasses, and a N95 respirator will also be worn when embryos are dosed or washed.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:
n/a

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Inject embryos, perform BBB and lateral line studies, obtain tissue for knockout verification or T3/T4 quantification. Make stocks and working solutions, dose embryos, wash embryos, assess larvae for developmental and neurobehavioral toxicity	7 years experience using zebrafish as a model for developmental toxicity. Has taken all appropriate lab, animal, and barrier training. Completed all NHEERL required training.
Exemption 6	Associate Principal Investigator	Make stocks and working solutions, dose embryos, wash embryos, assess larvae for developmental and neurobehavioral toxicity.	10 years experience using zebrafish as a model for developmental toxicity. Has taken all appropriate lab, animal, and barrier training. Completed all NHEERL required training.
Exemption 6	Technical Staff	Make stocks and working solutions, dose embryos, wash embryos, assess larvae for developmental and neurobehavioral toxicity	2 years experience using zebrafish as a model for developmental toxicity. Has taken all appropriate lab, animal, and barrier training. Completed all NHEERL required training.
Exemption 6	Technical Staff	ORISE contractor. Collect embryos, bleach embryos, and perform exposures, behavior, and basic assessments. Extract nucleic acids.	2 years basic laboratory experience at NCSU. Experience with sterile cell culture technique. Took a molecular biology lab course at NCSU that utilized zebrafish embryos. . Completed all NHEERL required training. Will work with Exemption 6 until basic proficiency established.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year n/a

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Zebrafish larvae will generally be euthanized on day 6. Larvae may occasionally be euthanized prior to Day 6. In the rare event that the behavioral equipment malfunctions, we may opt to keep larvae past Day 6. If this occurs, all larvae will be fed rotifers and/or artemia. All larvae will be euthanized by 14 days post fertilization

2. Describe the euthanasia techniques:

Method(s): Anesthesia

Agent(s): Two step procedure.

Primary methods: Ice cold buffered tricaine until larvae are anesthetized or rapid cooling until larvae are anesthetized (where no ice will be in contact with the larvae) or clove oil (eugenol) until larvae are anesthetized.

Secondary methods: Once larvae are anesthetized (no heartbeat visible), then maceration OR 1:5 bleach immersion OR immersion in fixative OR flash freezing in liquid nitrogen will be used to ensure death.

Dose (mg/kg): Eugenol 600-1500 ul/L, Tricaine 300- 900 ul/L; both of these are used via immersion until no heartbeat is seen.

Volume: Immersion.

Route: Immersion.

Source(s) of information used to select the above agents/methods:

Personal experience, veterinary staff, AVMA guidelines 2013, 2010 NIH guidelines and JAALAS 2015 Jan; 54(1): 76-79.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

Currently, the approved AVMA method for euthanasia of embryos or zebrafish larvae 4-7 days post fertilization is rapid chilling for at least 20 minutes following loss of operculum movement, followed by bleach.

We request an exception to the approved AVMA method due to our lab's experience of four years using rapid chilling on zebrafish embryos and larvae, and also due to specific experimental requirements for PCR or high throughput sequencing on nucleic acids on embryos and larvae after euthanasia. Bleach would preclude the use of PCR techniques and so we require another method of larvae euthanasia. In our hands, we have found by observing thousands zebrafish larvae, that the larvae have no heartbeat and are non-responsive as with general anesthesia after approximately 10 seconds in iced buffer. We will perform the second, physical method of

euthanasia immediately upon loss of heartbeat/ responsiveness to stimuli, while the larvae are anesthetized. These include; either immersion in 1:5 bleach, fixative, maceration, or flash freezing in liquid nitrogen.

At the end of these experiments, embryos or larvae will be euthanized either by rapid cooling as above until anesthetized (no heartbeat) followed by 1:5 bleach, or rapid cooling until anesthetized (no heartbeat) followed by maceration in PCR solution, sequencing tissue preparation solution, or flash freezing in liquid nitrogen. For maceration, no zebrafish embryo or larvae specific macerator is available. Either a homogenizer (probe or bed) or probe sonicator will be used.
Flash freezing in liquid nitrogen will be used to allow for the quantification of thyroid hormones. The extraction procedure will be carried out by the Exemption 6 Exemption 6 lab in NHEERL/TAD. Because we need to store tissue prior to extractions, tissue must be flash frozen in liquid nitrogen according to EPA publications "2 Inhibition of TPO I – Fathead minnow…pdf and 3 Inhibition of TPO II – zebrafish…pdf", attached below. These methods were based on the original 2004 Crane et al. paper (attached). All larvae will be anesthetized prior to flash freezing.

4. Describe how death is to be confirmed.
Death will be confirmed by use of either 1:5 bleach solution immersion or maceration or flash freezing in liquid nitrogen.

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Larvae will be placed in 1:5 bleach, liquid nitrogen, or macerated. Remaining liquid will be disposed of in hazardous waste containers located in chemical fume hoods in **Exemption 6** .
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6	12/07/2015

Exemption 6

Submitted: 12/14/2015

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	01/04/2016	Exemption 6	ISTD	MD
	by Exemption 6 /RTP/USEPA/US	***Lotus Notes Address*** Exemption 6 /RTP/USEPA/US	***Branch*** SBB	***Submitted to Branch Chief for Approval*** 12/14/2015 09:27 AM

ATTACHMENTS

19-01-001_response to prescreen.docx 19-01-001 Prescreen PI response.pdf

Crane et al_T3T4 extraction in fathead_1-s2.0-S001664800400187X-main.pdf

3 Inhibition of TPO II - zebrafish AQTOX-S-15-00602.pdf

2 Inhibition of TPO I - Fathead minnow_AQTOX-S-15-00634.pdf

Jomma et al. altex_2014_3_303_317__4f_Jomaa1.pdf OSU Toxcast supp toxsci_13_0620_File009.pdf

20151209_Thyroid LAPR # of animals.pdf 20151207_thyroid design.pdf [attachment "20151207_Thyroid LAPR # of animals for F0 studies.pdf" deleted by Exemption 6 RTP/USEPA/US] HSRP 578 Chem Screen.xlsx

Mundy HSRP list of chemicals.docx [attachment "20151127_Thyroid LAPR # of animals.pdf" deleted by Exemption 6 /RTP/USEPA/US] [attachment "20151127_Thyroid LAPR # of animals.pdf" deleted by Exemption 6 /RTP/USEPA/US] [attachment "20151125_thyroid design.pdf" deleted by Exemption 6 /RTP/USEPA/US]

High-throughput gene targeting and phenotyping in zebrafish using CRISPR Cas9 nbt.2842.pdf

Rapid reverse genetic screening using CRISPR in zebrafish.pdf 20151124_ _TH target list.xlsx

Actions

First Update notification sent: 03/02/2017
Second Update notification sent: 04/03/2017
First 2nd Annual notification sent: 03/02/2018
Second 2nd Annual notification sent: 03/02/2018
1st Expiration notification sent:
2nd Expiration notification sent:

History Log: